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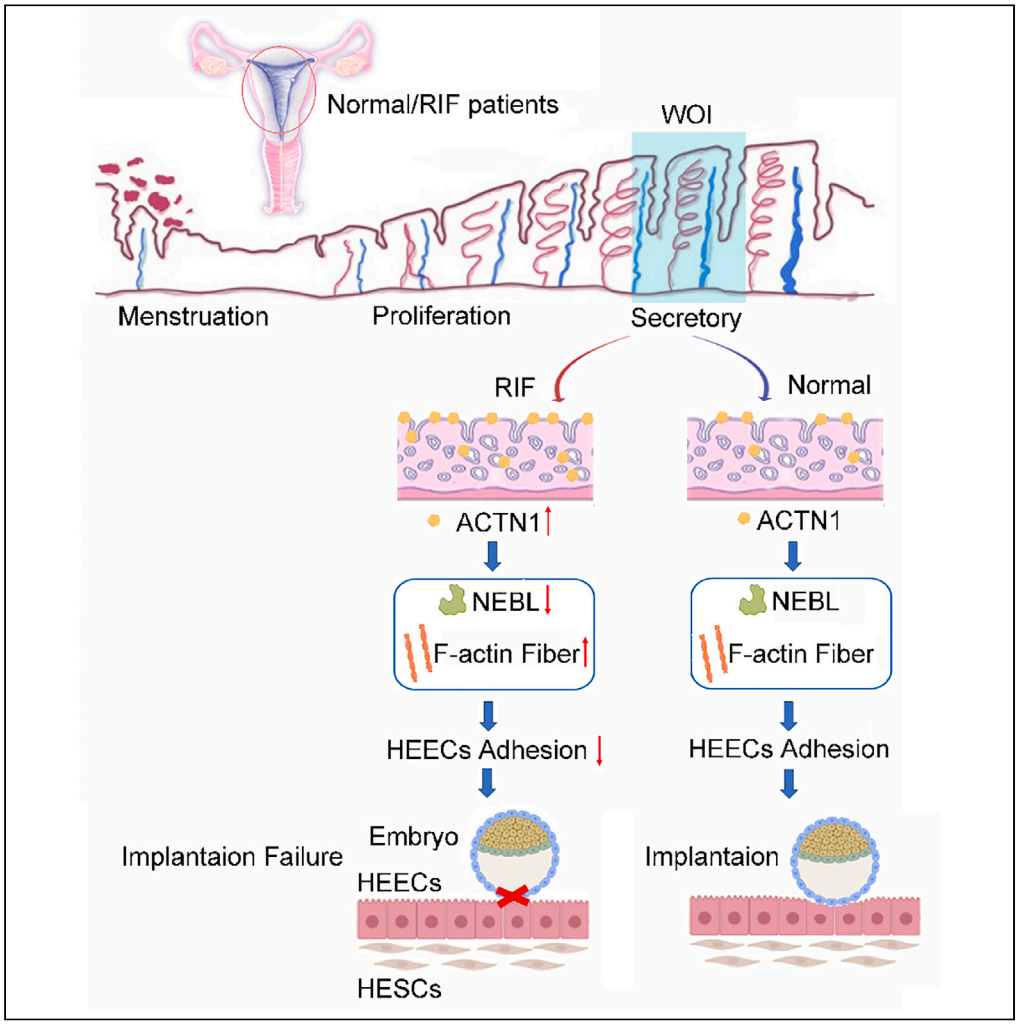
Upregulated α-actinin-1 impairs endometrial epithelial cell adhesion by downregulating NEBL in recurrent implantation failure



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Highlights

Poor endometrial receptivity is an obstacle to a successful pregnancy

Patients with recurrent implantation failure have an increased α-actinin-1 expression

α-actinin-1 overexpression significantly decreased nebulette levels

α-actinin-1 overexpression enhanced F-actin fiber levels and inpaired cell adhesion

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Upregulated α-actinin-1 impairs endometrial epithelial cell adhesion by downregulating NEBL in recurrent implantation failure

Xiaowei Zhou,[1,2] Jingru Duan,[1,2] Wenjie Zhou,[1] Aijun Zhang,[1,*] and Qian Chen[1,3,*]

SUMMARY

Poor endometrial receptivity results in embryo implantation failure. Acquisition of endometrial receptivity involves substantial structural alterations in the cytoskeleton and plasma membrane of epithelial cells, which facilitate embryo adhesion. However, the underlying molecular mechanism remains largely unknown. In this study, we identified that α-actinin-1 (ACTN1) was significantly downregulated in the mid-secretory phase of the endometrium compared with other phases; however, ACTN1 significantly increased in women with recurrent implantation failure (RIF). In Ishikawa and human endometrial epithelial cells (HEECs), ACTN1 overexpression significantly decreased NEBL levels, enhanced F-actin fiber levels, and caused a notable impairment in blastocyst adhesion, which mimicked the process of embryo adhesion. However, NEBL overexpression notably restored adhesion. Moreover, NEBL expression was reduced in patients with RIF compared with that in controls. Finally, our data showed that ACTN1 upregulation impaired endometrial receptivity in women with RIF, possibly by regulating NEBL expression and subsequent cell-adhesion capability.

INTRODUCTION

Poor endometrial receptivity is a big obstacle to a successful pregnancy.[1] Developing a receptive endometrium is indispensable for successful embryo implantation and subsequent placenta formation.[2,3] Mounting evidence shows that aberrant gene expression results in low endometrial receptivity, preventing the endometrium from responding to the embryo.[4] Kara et al. showed that the expression of Homeobox A10, Homeobox A11, and leukemia inhibitory factor (LIF) could be used to evaluate endometrial receptivity in patients with polycystic ovary syndrome patients (PCOS).[5] Despite significant advances in our understanding of the interactive processes between the uterus and blastocysts, the molecular mechanisms responsible for the establishment of endometrial receptivity are still unclear.

We previously identified 317 differentially expressed genes (DEGs) between the mid-secretory (MS) and proliferative phases of the uterus in healthy women of childbearing age.[6] Gene enrichment analysis revealed that cytoskeleton-related proteins were downregulated during the implantation window. Of these proteins, non-muscular α-actinin-1 (ACTN1) expression was downregulated in the MS phase compared with the proliferative phase in a normal endometrium. ACTNs are ubiquitously conserved actin-linking proteins that perform crucial non-muscle-related functions, including cell adhesion, migration, and cytokinesis.[7,8] Four identified ACTN family members exist, which are ACTN1–4, of which ACTN1 and ACTN 4 are expressed in various non-muscle cell types.[9] ACTN4 has been implicated in tumorigenesis, epithelial-mesenchymal transition, and cancer metastasis.[10] Recently, the role of ACTN1 in cancer progression was revealed. Oroxylin-A represses ACTN1 expression, which results in the inactivation of tumor-associated fibroblasts and subsequent suppression of breast cancer metastasis,[11] indicating that ACTN1 acts as an oncogene. Upregulation of ACTN1 destabilizes E-cadherin-based adhesion, thus enhancing the migratory potential of breast cancer cells.[12] However, the role of ACTN1 in endometrial receptivity remains unclear. Blastocyst implantation involves cell localization, adhesion, and migration, which are closely related to the structure and function of the cytoskeleton.[13] Given the importance of the actin cytoskeleton in blastocyst implantation and the fact that ACTN1 is an actin filament-linking protein, we speculated that aberrant ACTN1 expression might affect uterine endometrial receptivity.

This study aimed to evaluate ACTN1 expression in the endometrium of patients with recurrent implantation failure (RIF) and compared it with that in healthy controls. Furthermore, using primary human endometrial epithelial cells (HEECs) and Ishikawa cells, we aimed to investigate the effects of ACTN1 overexpression on F-actin levels, cell migration, and cell adhesion.

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Table 1. Clinical characteristics of women enrolled in the indicated phases of the menstrual cycle

	EP (n = 13)	LP (n = 13)	MS (n = 16)	p value
Age (year)	29.07 ± 3.45	31.00 ± 4.08	29.56 ± 3.98	0.422
BMI (kg/m^2)	20.94 ± 2.73	22.44 ± 3.03	21.45 ± 1.80	0.311
Basal FSH level (mIU/mL)	7.53 ± 1.61	7.29 ± 2.30	6.91 ± 1.92	0.692
Basal LH level (mIU/mL)	4.81 ± 2.72	4.79 ± 2.19	3.82 ± 1.50	0.368
Basal E_2 level (pg/mL)	42.69 ± 18.89	44.85 ± 22.49	37.25 ± 14.20	0.524
Basal P_4 level (pg/mL)	0.58 ± 0.41	0.75 ± 0.55	0.67 ± 0.36	0.600
Thickness of endometrium before ovulation (mm)	11.46 ± 1.98	10.23 ± 2.09	10.94 ± 1.91	0.297

The data are expressed as the mean ± SD and were analyzed using analysis of variance (ANOVA). BMI, body mass index; E_2, estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone; P_4, progesterone; EP, early proliferative phase; LP, late proliferative phase; MS, mid-secretory phase.

RESULTS

α-actinin-1 expression in endometrial tissues during the menstrual cycle

To investigate physiological changes in ACTN1 expression in the human endometrium, endometrial specimens were obtained from 42 healthy women during different menstrual cycle phases. The clinical characteristics of the control during their menstrual cycle phase are summarized in Table 1. No significant differences were observed among the three groups, as shown in Table 1. Western blot analysis revealed that ACTN1 was strongly expressed in the early proliferative (EP) and late proliferative (LP) phases; however, its expression was notably decreased in the MS phase (Figures 1A and 1B). This indicates that low ACTN1 expression contributes to embryo implantation. Immunofluorescence staining revealed that ACTN1 was localized in both epithelial and stromal cells in the MS phase, with deeper staining intensity in HEECs (Figure 1C).

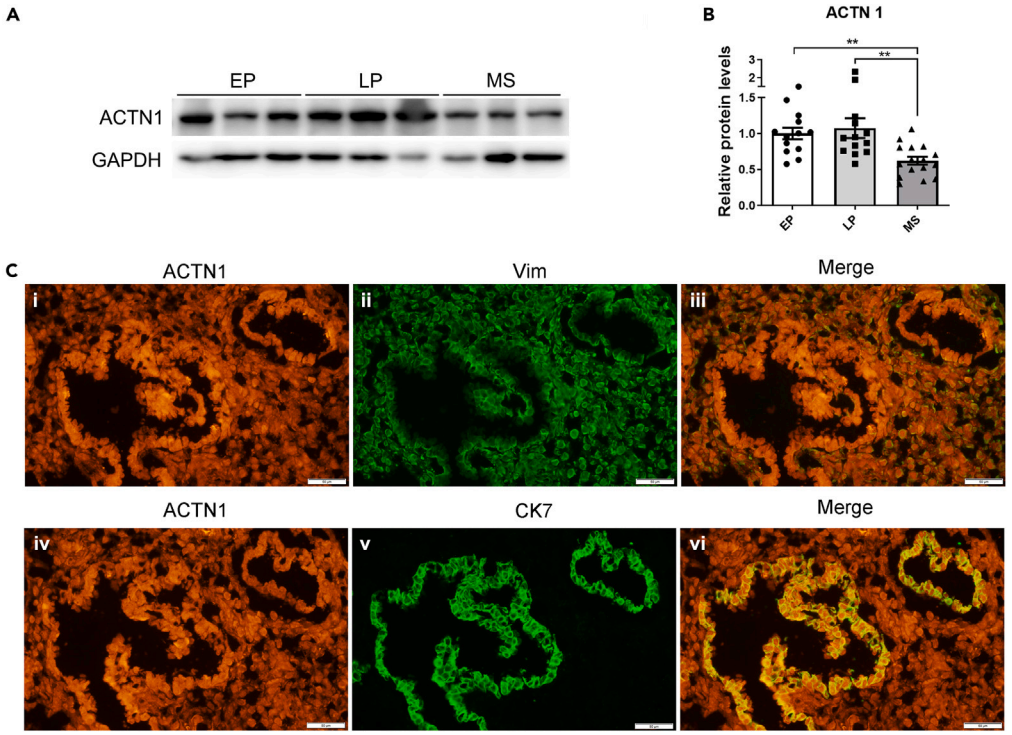


Figure 1. Expression of ACTN1 in different menstrual phases of women in the control group

(A) Representative western blot analysis.

(B) Densitometric quantification of ACTN1 in the endometrium during different menstrual phases of women in the control group. (EP, n = 13; LP, n = 13; MS, n = 16).

(C) Immunofluorescence co-localization staining of ACTN1 (i, iv), vimentin (ii), and cytokeratin 7 (v) were performed on the mid-secretory endometrium. The pictures were then merged (iii, vi). Original magnification, ×400. Scale Bar = 50 μm. Statistical analysis was performed using a one-way analysis of variance with the Bonferroni test. **p < 0.01. Data are presented as mean ± SEM. EP, early proliferative phase; LP, late proliferative phase; MS, mid-secretory phase; ACTN1, α-actinin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 2. Clinical characteristics of women in the control and repeated implantation failure groups

	Control (n = 12)	RIF (n = 13)	p value
Age (year)	29.92 ± 3.87	30.85 ± 4.41	0.582
BMI (kg/m^2)	21.78 ± 1.83	21.67 ± 1.96	0.885
Basal FSH level (mIU/mL)	6.90 ± 1.84	7.02 ± 1.45	0.854
Basal LH level (mIU/mL)	4.63 ± 2.09	4.00 ± 2.73	0.527
Basal E_2 level (pg/mL)	32.58 ± 16.45	44.30 ± 25.82	0.193
Basal P_4 level (pg/mL)	0.53 ± 0.31	0.77 ± 0.49	0.158
Thickness of endometrium before ovulation (mm)	11.00 ± 1.86	10.08 ± 1.50	0.183
Times of embryo transfers	1 (1,1)	4 (3,10)	<0.001
Number of blastocytes per transfer (%)			
1	7/12 (58.3%)	29/66 (43.9%)	0.358
2	5/12 (41.7%)	37/66 (56.1%)	
Proportion of high-quality blastocytes	9/17 (52.9%)	72/103 (69.9%)	0.167

Data are expressed as mean ± SD or frequency. The difference between the controls and patients with RIF was analyzed by independent samples t-test except "times of embryo transfers," which was calculated by independent samples Mann–Whitney U-test (median, range), and the "number of blastocytes per transfer" and "proportion of high-quality blastocytes," which were calculated by Pearson Chi-Square Continuity Correction. RIF, repeated implantation failure.

α-actinin-1 expression was increased in patients with recurrent implantation failure

Table 2 summarizes the demographic characteristics of the controls and patients with RIF. No significant differences were found between the groups, except for the number of embryo transfers. ACTN1 expression in the endometrium of patients with RIF was upregulated compared with that in the controls (Figures 2A–2D). The protein expression of LIF, a known marker of endometrial receptivity, was significantly lower in the RIF group than in the control group (Figures 2A and 2B). The western blot results show that ACTN1 expression was reduced in the MS

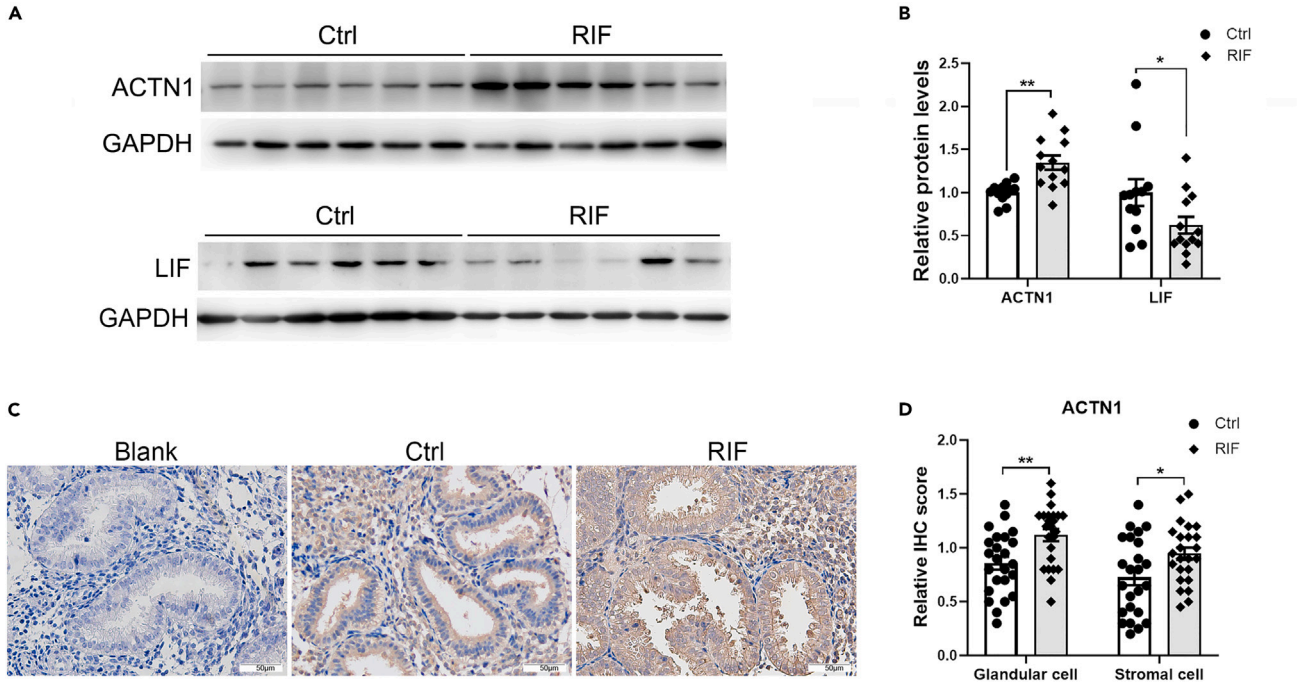


Figure 2. Aberrant expression of ACTN1 in the endometrium of patients with RIF

(A) Representative western blot analysis.

(B) Densitometric quantification of ACTN1/LIF in the Ctrl and RIF groups (N ≥ 12).

(C and D) Immunohistochemical staining and semi-quantification of ACTN1 in the mid-secretory phase (MS) phase endometrium of the women in the control and RIF groups. Original magnification, ×200. Scale Bar = 50 μm. Statistical analysis was performed using the Student's t test. *p < 0.05; **p < 0.01. Data are presented as mean ± SEM. LIF, leukemia inhibitory factor; Ctrl, healthy control group; ACTN1, α-actinin-1; MS, mid-secretory phase; RIF, recurrent implantation failure.

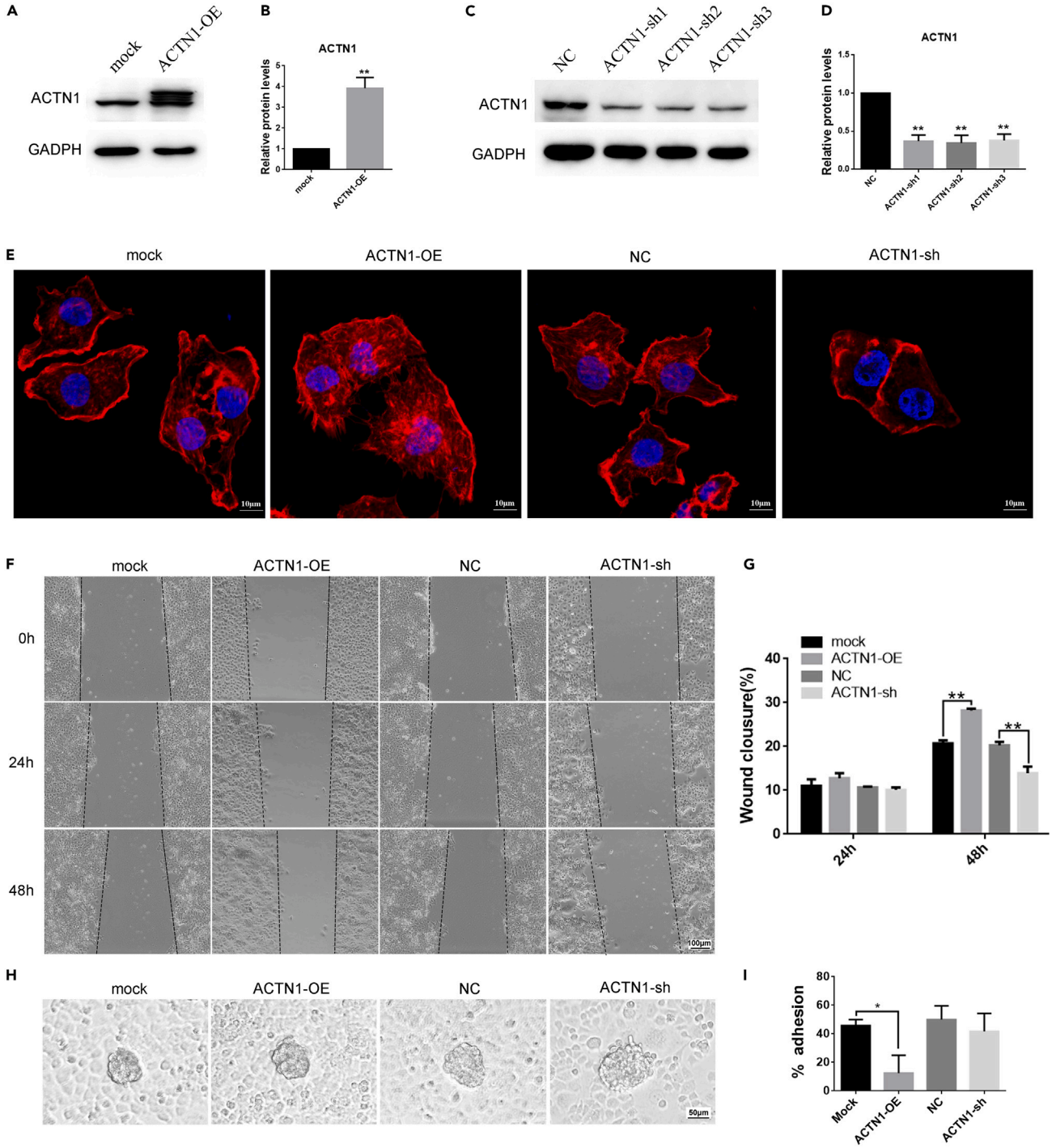


Figure 3. Effects of ACTN1 knockdown/overexpression on F-actin remodeling and cell migration and adhesion capability of Ishikawa cells

(A and B) Protein expression levels of ACTN1 were increased after ACTN1 overexpression (ACTN1-OE) in Ishikawa cells, as shown by western blot analysis.

(C and D) Protein expression levels of ACTN1 were decreased after ACTN1 silencing (ACTN1-sh) using three different ACTN1 shRNAs in Ishikawa cells, as shown by western blot and densitometric quantification.

(E) Localization of F-actin was detected in Ishikawa cells with either ACTN1 overexpression or knockdown by rhodamine-phalloidin staining.

(F and G) Observations and results of the wound-healing assay performed on Ishikawa cells transfected with mock plasmid, ACTN1 plasmids, NC shRNA, or ACTN1 shRNA. Wound width was measured at 0, 24 and 48 h after wounding.

Figure 3. *Continued*

(H and I): Effects of ACTN1 on the adhesion capability of Ishikawa cells using "embryo-endometrial" adhesion model. The blastocyst attachment rate was calculated. Significance was determined using Student's *t* test (Figures 3B, 3G, and 3I) and one-way ANOVA with the Bonferroni test (Figure 3D), respectively. *p < 0.05, **p < 0.01. Data are presented as mean ± SEM. NC shRNA, negative short hairpin RNA; ACTN1, α-actinin-1; shRNA, short hairpin RNA; ANOVA, analysis of variance.

phase compared with that in the EP/LP phase in the endometrium of healthy women. However, ACTN1 expression in the MS phase aberrantly increased in patients with RIF. These data suggest that ACTN1 expression correlates with endometrial receptivity.

α-actinin-1 overexpression affected F-actin levels, migration ability, and adhesion capability in Ishikawa cells and human endometrial epithelial cells

Given that ACTN1 is a cytoskeletal protein that crosslinks actin filaments, and HEECs must undergo marked morphological alterations to promote embryo implantation, we further explored whether the aberrant ACTN1 expression altered the cytoskeleton. As shown in Figure 1C, ACTN1 was mainly expressed in HEECs and minimally expressed in endometrial stromal cells. We further assessed whether ACTN1 affected the organization of the actin cytoskeleton in HEECs by exploring the localization of F-actin using rhodamine-phalloidin staining. Both knockdown- and overexpression-based approaches were used in Ishikawa cells (Figures 3A–3D). An apparent increase in F-actin fiber levels was observed when ACTN1 was overexpressed, whereas reduced F-actin levels were observed upon ACTN1 knockdown in Ishikawa cells (Figure 3E). Subsequently, we investigated the functional characteristics of ACTN1, with an emphasis on its role in regulating cell migration and adhesion. The results of the migration assay revealed that ACTN1 overexpression significantly increased Ishikawa cell migration, which was inhibited following ACTN1 knockdown (Figures 3F and 3G). ACTN1 overexpression inhibited the adhesion capability of Ishikawa cells, demonstrated by a heterologous "embryo-endometrial" adhesion model, whereas ACTN1 knockdown did not affect the adhesion capability of Ishikawa cells (Figures 3H and 3I).

Cultured primary HEECs were identified using immunofluorescence staining for cytokeratin 7 (Figure 4A). Both knockdown- and overexpression-based approaches were used for HEECs (Figures 4B–4E). Significantly increased F-actin fiber levels were observed when ACTN1 was overexpressed, whereas reduced F-actin levels were observed upon ACTN1 knockdown in HEECs (Figure 4F). Furthermore, ACTN1 overexpression inhibited the trophoblast adhesion capability of HEECs, whereas ACTN1 knockdown had no significant effect on the adhesion capability (Figures 4G and 4H).

RNA-seq analysis of α-actinin-1-overexpressed Ishikawa cells

To explore the molecular mechanisms underlying ACTN1 in cytoskeletal regulation, ACTN1 was overexpressed in Ishikawa cells and subjected to RNA-seq analysis. A total of 319 differentially expressed mRNAs (including 145 upregulated and 174 downregulated mRNAs) were identified between the ACTN1-overexpressed and control groups (Figure 5A, $p < 0.05$). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of these DEGs indicated that these dysregulated genes were primarily concentrated in extracellular structure organization, extracellular matrix components, extracellular matrix organization, MHC protein complex, proteinaceous extracellular matrix, integrin binding, and cell adhesion molecule binding (Figures 5B and 5C). We validated the selected DEGs from the RNA-seq results using RT-qPCR (Figure 5D).

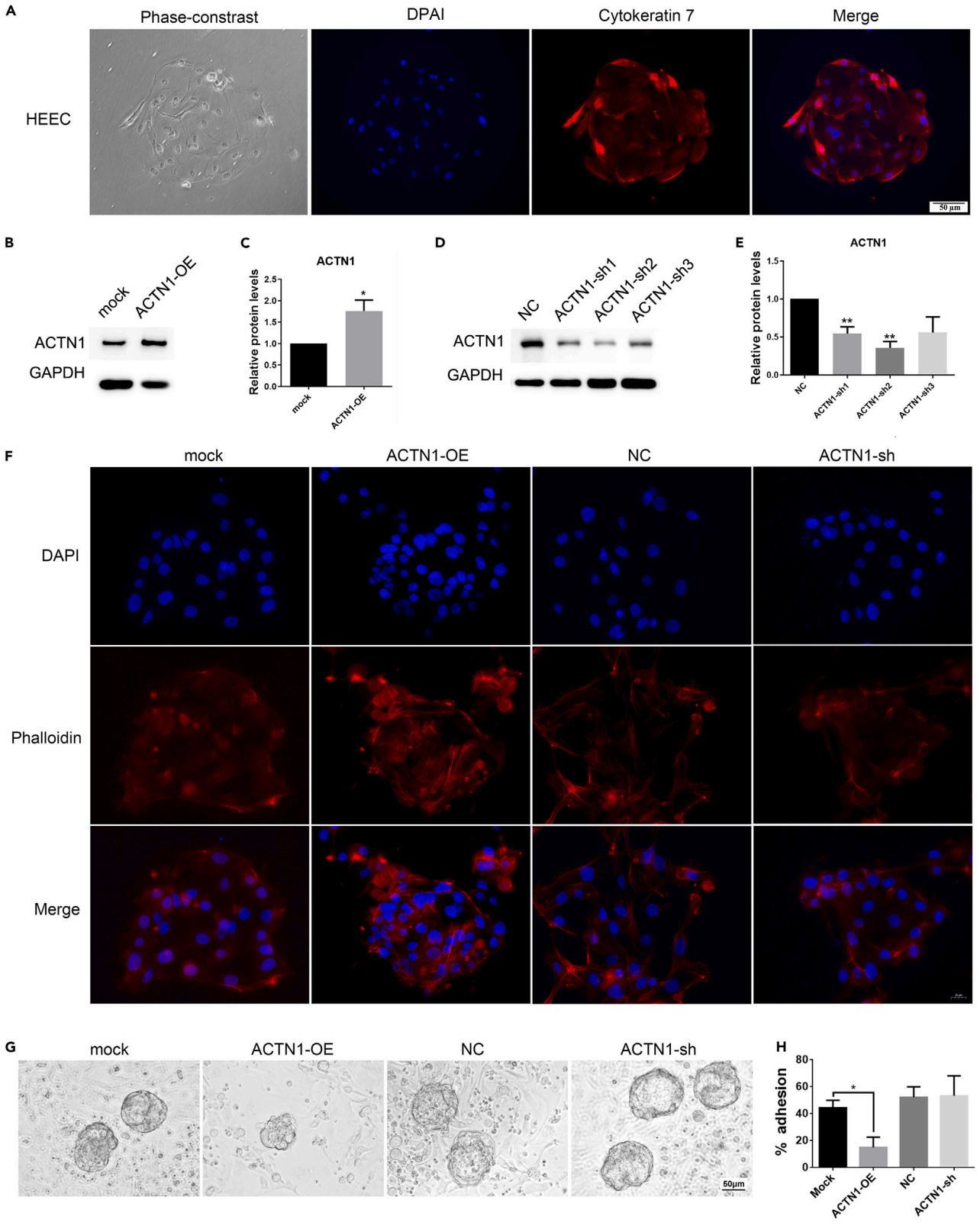
Among these genes, NEBL, a crucial regulator of cytoskeletal dynamics and actin filament stability, was dramatically downregulated in Ishikawa cells after ACTN1 overexpression. To further verify the regulatory role of ACTN1 in NEBL expression, qPCR was performed to assess the mRNA levels of NEBL in Ishikawa cells following ACTN1 overexpression. Figure 5D shows that NEBL mRNA levels markedly decreased after ACTN1 overexpression. More importantly, NEBL expression was lower in patients with RIF than in controls (Figure 6).

α-actinin-1-mediated NEBL expression was involved in the regulation of human endometrial epithelial cells and Ishikawa adhesion capability

In HEECs and Ishikawa cells, NEBL levels decreased after ACTN1 overexpression (Figures 7A and 7B). Intrauterine injection of lentiviruses successfully infected the mice endometrial epithelial cells (infected cells showed GFP expression), ACTN1 expression was detected via immunohistochemistry suggests that the construction of ACTN1-knockdown endometrial epithelial cells via the transfection of EGFP-ACTN1-sh lentivirus into mice endometrium was successful and the level of NEBL and endometrial receptivity marker LIF increased following ACTN1 knockdown (Figure S1). The "Embryo-endometrial" adhesion assay showed that the declined adhesion capability in ACTN1 overexpression HEECs could be partially reversed by NEBL overexpression (Figures 7C and 7D). These data confirm that upregulated ACTN1 impairs endometrial receptivity, probably by regulating NEBL expression and the subsequent adhesion capability of human endometrial epithelial cells.

DISCUSSION

In this study, we demonstrated that (i) ACTN1 expression was upregulated in the MS endometrium of patients with RIF; (ii) ACTN1 overexpression increased F-actin levels but inhibited cell adhesion capability in both Ishikawa cells and primary HEECs; (iii) ACTN1 repressed NEBL expression, and NEBL expression was downregulated in the endometrium of patients with RIF; and (iv) the inhibitory effects of ACTN1


A
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NC
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Phalloidin
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G
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ACTN1-OE
NC
ACTN1-sh
50μm
H
% adhesion
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ACTN1-OE
NC
ACTN1-sh

Figure 4. Effects of ACTN1 knockdown/overexpression on F-actin remodeling and adhesion capability in HEECs

(A) Immunofluorescence staining of Cytokeratin 7 in HEECs.

(B and C) Protein expression levels of ACTN1 were increased after ACTN1 overexpression (ACTN1-OE) in HEECs, as shown by western blot and densitometric quantification (GAPDH).

(D and E) Protein expression levels of ACTN1 were decreased after ACTN1 silencing (ACTN1-sh) in HEECs, as shown by western blot analysis.

(F) Localization of F-actin was detected in HEECs with either ACTN1 overexpression or knockdown by rhodamine-phalloidin staining.

(G and H) Effects of ACTN1 on the adhesion capability of primary HEECs by "embryo-endometrial" adhesion model. The blastocyst attachment rate was calculated. Scale Bar = 50 μm. Significance was determined using Student's *t* test (C and 4H) and the one-way ANOVA with the Bonferroni test (E), respectively. *p < 0.05, **p < 0.01. Data are presented as mean ± SEM. HEECs, human endometrial epithelial cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ACTN1, α-actinin-1; ANOVA, analysis of variance.

overexpression on HEECs adhesion capability could be efficiently rescued by NEBL overexpression. These results reveal the important roles of ACTN1 and NEBL in regulating endometrial receptivity and may provide insights into the treatment of infertility.

To promote embryo implantation, the endometrium undergoes marked morphological and functional alterations, accompanied by adaptive variations in gene expression. Endometrial epithelial cells (EECs) are the first site of contact between the uterus and invading blastocyst.[14] To promote pregnancy, HEECs undergo a transition from non-receptive to receptive, including plasma membrane transformation throughout the basal, lateral, and apical membranes, which results in the disruption of the cytoskeleton.[15] These morphological changes ultimately lead to a reduction in cell polarity and an increase in apical adhesiveness, which contribute to embryo attachment.[16] Increasing evidence suggests that the reorganization of the actin cytoskeleton plays a key role in regulating cell morphology and functional alterations.[16–18] Moreover, few studies have investigated the cytoskeletal regulators during epithelial transformation at the onset of uterine receptivity. In this study, we found that ACTN1, an actin cross-linking protein, was mainly expressed in EECs. The results also revealed that ACTN1 was strongly expressed in the endometrium of healthy women during both the EP and LP phases but was significantly decreased in the MS phase. Moreover, ACTN1 expression was aberrantly increased in patients with RIF compared with the controls during the MS phase. The opposite trend was observed for the expression of LIF, which is an accepted marker of endometrial receptivity. These results suggest that ACTN1 upregulation in EECs during the window phase impairs endometrial receptivity.

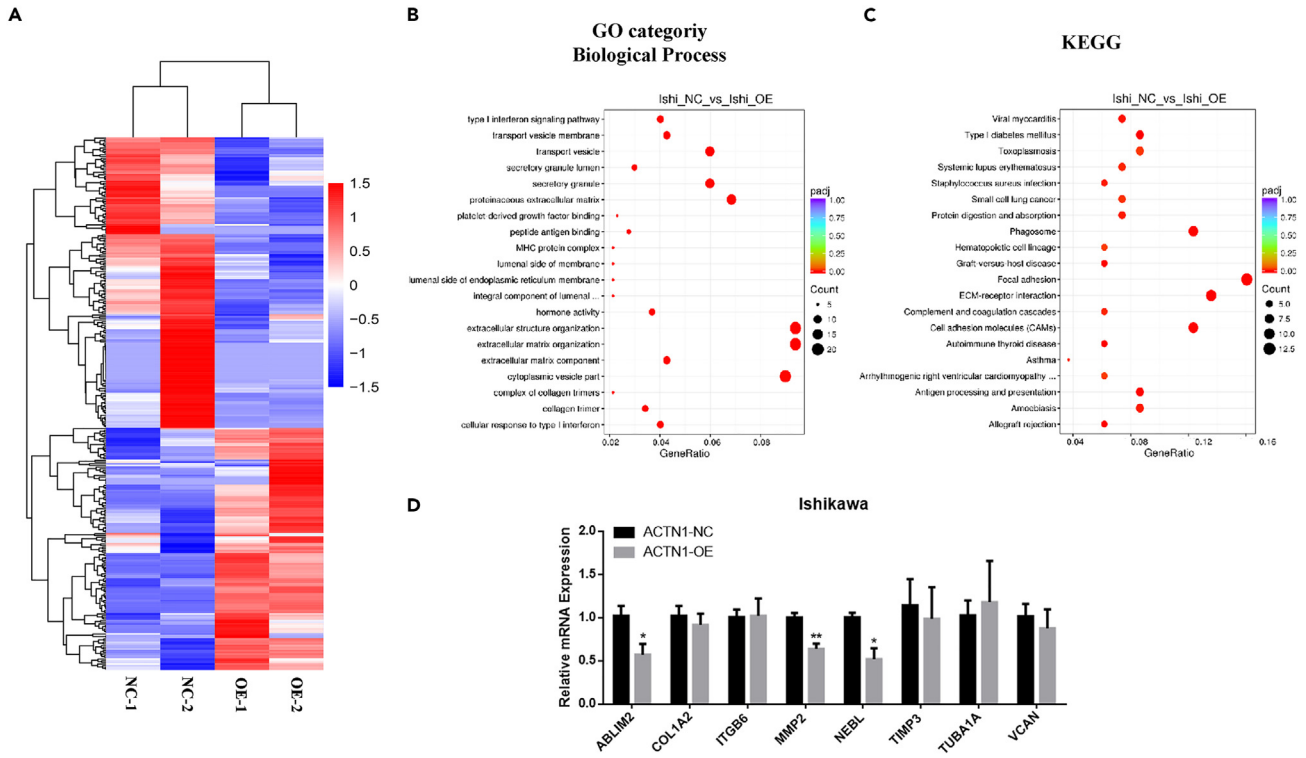

Figure 5. Analysis of transcriptome alteration in ACTN1 overexpression Ishikawa cells

(A) Hierarchical clustering of DEGs. Functional enrichment analysis of DEGs, including (B) Gene Ontology, and (C) Kyoto Encyclopedia of Genes and Genomes pathway.

(D) Representative DEGs were validated by RT-qPCR. Significance was determined using Student's *t* test. *p < 0.05; p < 0.01. Data are presented as mean ± SEM. DEGs, differentially expressed genes; RT-qPCR, reverse transcription-quantitative polymerase chain reaction. ACTN1, α-actinin-1.

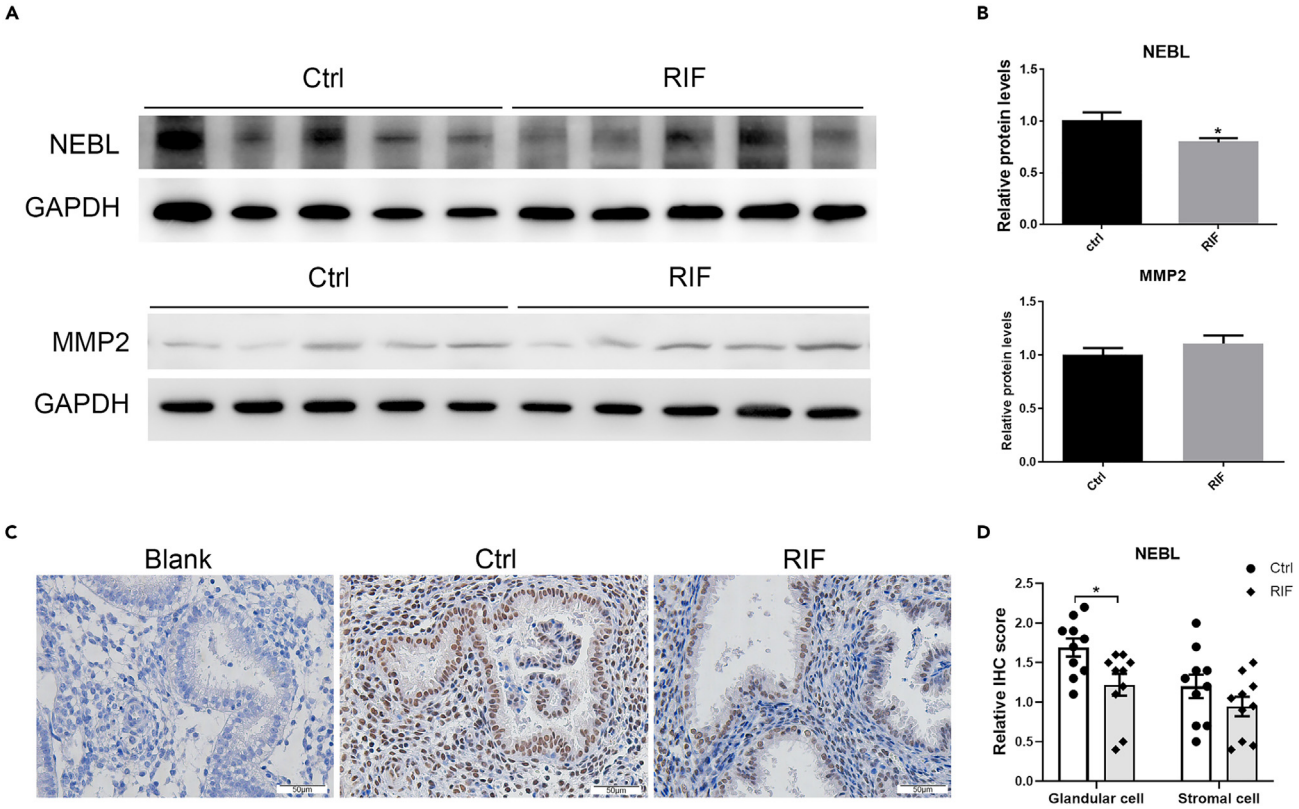

Figure 6. Expression of NEBL in the Ctrl and RIF groups

(A) Western blot analysis (GAPDH).

(B) densitometric quantification of NEBL and MMP2 in the endometrium of the women in the Ctrl and RIF groups.

(C and D) Immunohistochemical staining and semi-quantification of NEBL in the endometrium of the women in the Ctrl and RIF groups. Original magnification, × 200. Scale Bar = 50 μm. Significance was determined using Student's *t* test. *p < 0.05. Data are presented as mean ± SEM. NEBL, Nebulette; Ctrl, control; RIF, recurrent implantation failure; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP2, matrix metallopeptidase.

ACTN1–4 are major cytoskeletal proteins that perform important non-muscle functions, including epithelial-mesenchymal transition, cytokinesis, cell migration, and cell adhesion.[7,19,20] ACTN1 role in regulating cancer cell migration and adhesion has been previously verified. Xie et al. revealed that high ACTN1 expression correlates with poor prognosis in oral squamous cell carcinomas and that ACTN1 knockdown represses cancer cell migration.[7] In breast cancer cells, ACTN1 overexpression facilitates cell migration accompanied by E-cadherin-based adhesion and reorganization of the actin cytoskeleton.[12] Additionally, ACTN1 is associated with cell-matrix adhesion organization in keratinocytes.[21] However, the correlation between aberrant ACTN1 expression, EEC migration, and adhesion remains unknown. In this study, we demonstrated that increased ACTN1 expression resulted in significantly increased F-actin fiber levels, whereas reduced ACTN1 expression repressed F-actin levels in Ishikawa cells and HEECs. ACTN1 overexpression significantly increased Ishikawa cell migration, whereas ACTN1 knockdown suppressed this effect. The role of ACTN1 in the pro-migratory capabilities of EECs is consistent with previous observations in cancer cells; however, conflicts with previous conclusions that EEC migration contributes to endometrial receptivity exist. We hypothesized that ACTN1 may cause "hyper-migration" of HEECs, resulting in the subsequent impairment of endometrial receptivity. Adhesion experiments confirmed the adverse effects of ACTN1 overexpression on embryonic adhesion. Implantation is the initial stage of pregnancy in which stable adhesion between the embryo and maternal tissue needs to be established.[22] Using hatched mouse blastocyst adhesion assays, we found that ACTN1 overexpression inhibited the adhesion capacity of Ishikawa cells and primary HEECs. Based on the results of this study, ACTN1 overexpression may lead to the abnormal migration and adhesion of HEECs by altering the cytoskeleton, thus damaging endometrial receptivity and causing failure of embryo implantation.

The endometrium undergoes adaptive variations in gene expression to facilitate embryo implantation. To identify the genes regulated by ACTN1 in HEECs, ACTN1 was overexpressed in Ishikawa cells, and the DEGs were analyzed using RNA-seq. The RNA-seq results showed that 319 mRNAs were differentially expressed following ACTN1 overexpression. Bioinformatics analysis revealed that dysregulated genes were mainly enriched in extracellular structure organization, extracellular matrix components, extracellular matrix organization, MHC protein complex, proteinaceous extracellular matrix, integrin binding, and cell adhesion molecule binding. This study further verified that NEBL, a crucial regulator of cytoskeletal dynamics and actin filament stability, was repressed by ACTN1.

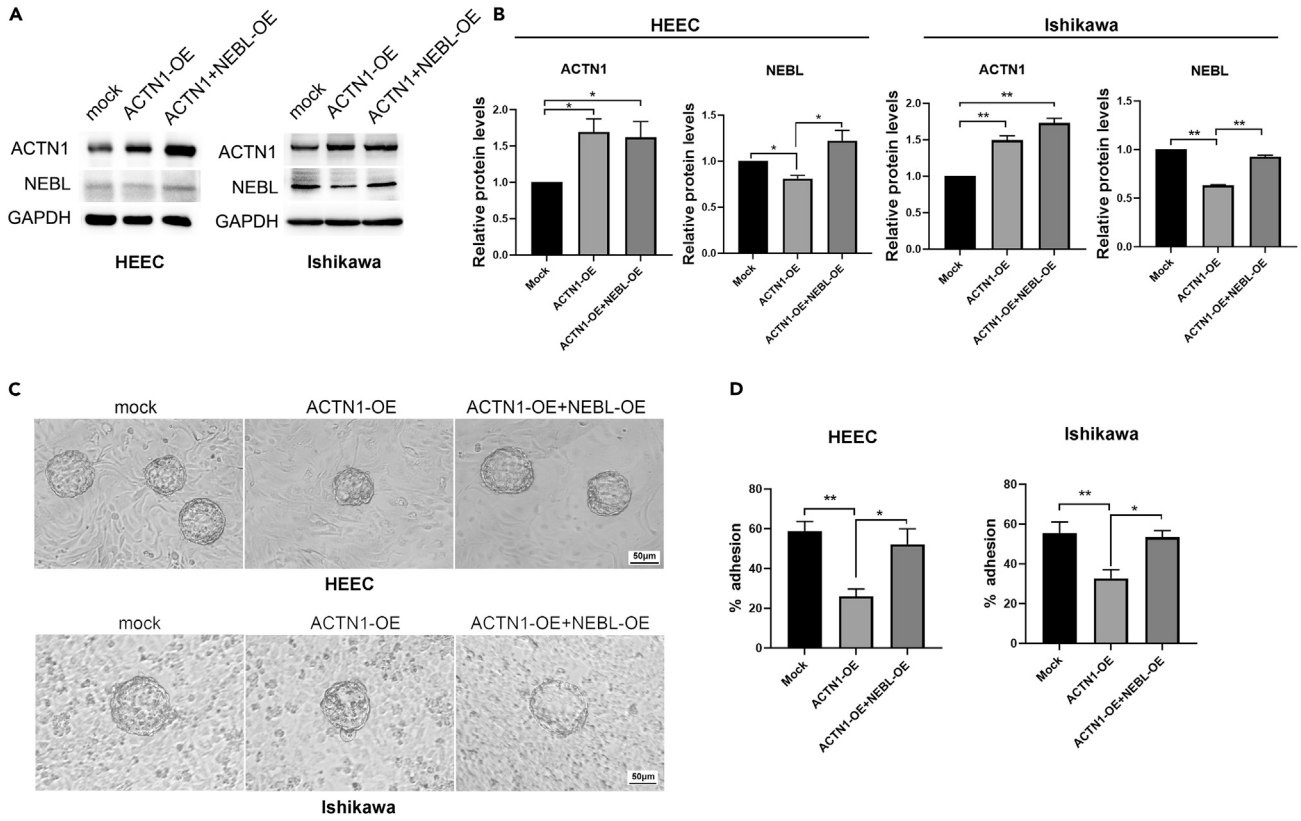

Figure 7. ACTN1-mediated NEBL expression was involved in the regulation of human endometrial epithelial cell adhesion capability

(A and B) NEBL expression in HEECs and Ishikawa cells transduced with control, ACTN1 plasmid or NEBL plasmid.

(C and D) Effects of ACTN1 and NEBL on the adhesion capability of primary HEECs and Ishikawa cells by "embryo-endometrial" adhesion model.

Scale Bar = 50 μm. *p < 0.05; p < 0.01. Data are presented as mean ± SEM. ACTN1, α-actinin-1; NEBL, Nebulette; HEECs, human endometrial epithelial cells.

NEBL belongs to the nebulin family of actin-binding proteins that are highly conserved and play vital scaffolding functions in the cytoskeleton and focal adhesion dynamics.[23] Owing to its abundant expression in cardiac muscles, NEBL has a significant effect on the regulation of cardiac function.[24] In cardiomyocytes under biomechanical stress, NEBL instantly translocates to focal adhesions and recruits crucial adhesion molecules.[23] However, NEBL has been detected in many other tissues[25] and plays an important role in actin filament architecture and cell adhesion.[26] NEBL can facilitate the interaction of several key focal adhesion components, making it an important scaffolding protein for cell adhesion in different cancer cells.[27] NEBL also plays a major role in the adhesion dynamics of podocytes; NEBL knockdown leads to the dysregulation of intermediate filament architecture and compromises focal adhesion,[28] suggesting that NEBL is closely related to actin filaments and adhesion function. In this study, we further verified that the expression of NEBL was negatively regulated by ACTN1 and that NEBL levels were downregulated in patients with RIF compared with the controls. As expected, the inhibitory effects of ACTN1 on cell adhesion were effectively reversed by NEBL overexpression. Altogether, the current data demonstrate that upregulated ACTN1 impairs endometrial receptivity, probably by regulating NEBL expression and the subsequent adhesion dynamics of HEECs.

Our results revealed an important role of ACTN1 in the regulation of endometrial receptivity. Little research has been conducted on the correlation between the cytoskeleton and endometrial receptivity in the ER. This study aimed to focus on the impact of cytoskeletal regulation on endometrial receptivity; no previous reports on the impact of ACTN1 on endometrial receptivity exist.

In summary, ACTN1 expression in the human endometrium was assessed. The results demonstrated that the upregulation of ACTN1 impaired HEECs adhesion capability, most likely by regulating NEBL expression and the subsequent adhesion dynamics of HEECs, which may ultimately affect endometrial receptivity. These results enhance our understanding of the molecular mechanisms underlying RIF and facilitate the development of new therapeutic targets.

Limitations of the study

This study has some limitations. Although we identified a regulatory correlation between ACTN1 and NEBL expression, the function of NEBL in ACTN1-regulating HEEC migration remains unclear. Therefore, further in-depth studies are required to analyze the specific mechanism of the ACTN1-NEBL-F-actin chain. In addition, although our study has experimentally demonstrated the effect of ACTN1 on endometrial receptivity in cultured cells, its mechanistic role requires further validation in animal models.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109046.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: X.Z., Q.C. and A.Z.; collected the clinical samples: W.Z., Q.C.; performed the experiments: X.Z., J.D.; analyzed the data: X.Z., J.D.; wrote the article: X.Z. and Q.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
ACTN1	R&D Systems	Cat #AF8279;
NEBL	Santa Cruz Biotechnology	Cat #sc-393784;
MMP2	Abcam	Cat #ab86607; RRID: AB_10672798
GAPDH	Cell Signaling Technology	Cat #2118; RRID: 10100147C
LIF	Abcam	Cat # ab138002; RRID: AB_3083551
cytokeratin 7	Abcam	Cat # ab181598; RRID: AB_2783822
Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594	Thermo Fisher Scientific	Cat # A-11012 RRID:AB_2534079
Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	Thermo Fisher Scientific	Cat # A-11001 RRID:AB_2534069
Bacterial and virus strains		
pHB-U6-MCS-CMV-ZsGreen-PGK-PURO	Hanbio (Shanghai, China)	N/A
The effective target sequences of ACTN1 shRNA	This manuscript	Table S2
Biological samples		
Human endometrial samples (Asian, China, Female, age: 25–35)	Reproductive Medical Center, Ruijin Hospital (Approval Number: 2012-57)	http://www.rjh.com.cn/2018RJPortal/szyxzx/index.shtml
Experimental models: Cell lines		
Human: Ishikawa cell line	ECACC	99040201
Experimental models: Organisms/strains		
C57BL/6 mice (8-week-old)	Beijing Vital River Laboratory Animal Technology Co., Ltd.	N/A
Chemicals, peptides, and recombinant proteins		
DMEM basic	Gibco	Cat #C11995500BT
FBS	Gibco	Cat # 10100147C
G-2 plus	Vitrolife	Cat # 10132
Collagenase	Thermo Fisher Scientific	Cat # 17100017
rhodamine-phalloidin	Thermo Fisher Scientific	Cat # A12381
DAPI	Roche	Cat # MBD0015
X-tremeGENE HP DNA Transfection Reagent	Roche	Cat # 06366236001
Recombinant DNA		
pEZ-M02	FulenGen (Guangzhou, China)	N/A
ACTN1 overexpression plasmids	This manuscript	N/A
NEBL overexpression plasmids	This manuscript	N/A
Oligonucleotides		
Primer sequences	This manuscript	Table S1
Software and algorithms		
ImageJ	NIH	https://imagej.nih.gov/ij/
GraphPad prism	GraphPad Software	https://www.graphpad.com/
SPSS	IBM	https://spss.en.softonic.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Qian Chen (cq_passion@163.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original codes.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Patients

All participants provided written informed consent for the use of human tissues. This study was approved by the Ethics Committee of Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University (number: 2012-57).

From February 2020 to November 2021, 25 women aged 25–35 years who visited the Reproductive Medical Center of Ruijin Hospital for *in vitro* fertilization (IVF) treatment were recruited for this study. Women with RIF (n = 13) and those with successful IVF cycles (control) (n = 12) were enrolled into two separate groups. The inclusion and exclusion criteria for both groups have been described previously.[29] Briefly, the control group included women with tubal obstruction factor-related infertility who had successfully achieved clinical pregnancy on their first attempt at embryo transfer. Women in the RIF group were unable to achieve a clinical pregnancy after transferring at least four good-quality embryos across a minimum of three fresh or frozen cycles. All transferable embryos were in the blastocyst stage, and embryos of grades exceeding 4 BC using the Gardner grading system were classified as high quality. From days 7–9 of the menstrual cycle, all the participants underwent transvaginal ultrasonography every other day until the dominant follicle diameter was 15–16 mm. Transvaginal ultrasonography was completed daily, and serum estrogen, luteinizing hormone (LH), and progesterone levels were measured daily until the follicle was discharged. Endometrial specimens were collected on day LH +7 (MS phase) using a pipe suction curettage (LILYCLEANER, China).

Based on the exclusion and inclusion criteria, 30 women with regular 28-day cycles were enrolled for the menstrual cycle analysis. The method for monitoring ovulation to confirm the menstrual period was the same as described above. On the day of endometrial collection, the specimens were divided into three groups: EP phase (days 5–7 of the menstrual cycle, n = 13), LP phase (days 11–13 of the menstrual cycle, n = 13), and MS phase (days 20–22 of the menstrual cycle, n = 16). The endometrial phase was identified by immunohistochemistry.

Moreover, to isolate primary endometrial cells, 12 MS-phase endometrial samples were collected from the women in the control group. Endometrial samples were collected as previously described.

METHOD DETAILS

Cell culture

Primary HEECs were isolated as previously described.[30] Briefly, fresh endometrial biopsy samples were rinsed with phosphate-buffered saline (PBS), finely minced into small fragments, and subsequently digested using 1 mg/mL collagenase type I (Thermo Fisher Scientific, MA, USA) in a 37°C shaker for 30 min. The mixture was sequentially passed through two sieves (from 100 μm to 40 μm sieves) (Millipore, Billerica, MA, USA), and the epithelial cells were retained on the 40 μm sieve. After washing with PBS, the filtrate was centrifuged at 100 × g for 5 min to collect primary HEECs. Immunofluorescence staining of cytokeratin 7 (ab181598, 1:100) was performed to identify the cultured primary HEECs. Ishikawa cells were purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK) and were cultured in DMEM/F12 containing 1% penicillin-streptomycin and 10% fetal bovine serum (Thermo Fisher Scientific) at 37°C in a humidified incubator under 5% CO_2 according to standard procedures.

Quantitative real-time PCR

The MiniBEST Universal RNA Extraction Kit (Takara, Beijing, China) was used to isolate total RNA from the samples following the manufacturer's instructions. The RNA was then reverse-transcribed using PrimeScript RT Master Mix (Takara). Gene expression analysis was performed using the SYBR Green Master Mix (Takara) and an Applied Biosystems 7500 Real-Time PCR System (Life Technologies (Applied Biosystems), CA, USA). Primer sequences used in these experiments are listed in Table S1. Relative quantification of the mRNA levels was performed using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene. All experiments were performed at least thrice.

Western blot

Endometrial samples and cells were lysed with RIPA buffer (Thermo Fisher Scientific) supplemented with a 1% protease inhibitor cocktail (Roche, Basel, Switzerland). The lysates were then centrifuged for 10 min at 4°C (12,000 × g) to collect the supernatants. Samples with equal amounts of proteins (30 μg) were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Subsequently, the proteins were transferred onto polyvinylidene fluoride membranes, followed by blocking, primary antibody incubation, appropriate secondary antibody incubation, and visualization. Primary antibodies against ACTN1 (Abcam, 1:1000), NEBL (Santa Cruz Biotechnology, 1:200), matrix metallopeptidase 2 (Abcam, 1:100), and GAPDH (Cell Signaling Technology, 1:1000) were used. Quantification of the results was performed by normalization to GAPDH using the ImageJ software.

Immunohistochemistry and immunofluorescence staining

Paraffin sections (5 μm) were prepared using standard procedures and were fixed. For immunohistochemistry, antigen retrieval was constructed by incubating the sections in buffered citrate (15 min, at 100°C). The sections were blocked using 5% w/v bovine serum albumin (30 min, 20°C–25°C) and then incubated overnight at 4°C with primary antibodies against ACTN1 (Abcam, 1:100) and NEBL (Santa Cruz, 1:50). Secondary antibodies were then added, followed by counterstaining with diaminobenzidine and hematoxylin. Specimens were examined under a BX53F microscope (Olympus, Tokyo, Japan). Immunohistochemical staining of each cell was evaluated by the intensity of staining, which ranged from 0 (negative staining) to 3 (the most intense staining). ACTN1 and NEBL immunohistochemistry was scored using the H-score calculation [(percentage at 1) × 1+ (percentage at 2)×2+ (percentage at 3) × 3].

For immunofluorescence analysis, Ishikawa cells and mid-secretory-phase HEECs were cultured in a 24-well chamber on glass coverslips and stained with rhodamine-phalloidin (Thermo Fisher Scientific, 1:100) for F-actin and DAPI (Roche) for nuclei. Sections were mounted with DAPI. The signals were visualized using a fluorescence microscope (Nikon, TS, Japan). Immunostaining was performed in triplicate.

Short-interfering RNA knockdown and plasmid overexpression studies

Negative control luciferase short hairpin RNA (shRNA) and shRNA targeting human ACTN1 were obtained from Hanbio (Shanghai, China). The effective target sequences of ACTN1 shRNA used in this study are presented in Table S2. FulenGen (Guangzhou, China) provided the mock ACTN1 overexpression plasmids and NEBL overexpression plasmids. The cells were transfected with shRNAs or plasmids as previously described.[30]

Whole-genome expression profile analysis

Whole-genome expression profiles of four mock and ACTN1 overexpression Ishikawa cell samples were investigated. Total RNA was extracted from the tissue by TRIzol Reagent (Thermo Fisher Scientific), and genomic DNA was eliminated by DNase I (TaKara). An RNA-seq transcriptome library was constructed using the TruSeq RNA sample preparation kit (Illumina, San Diego, CA, USA). Read mapping, principal component analysis (PCA), differential expression analysis, and functional enrichment analysis were performed using previously published methods.[31]

Wound-healing assay

Ishikawa cells, with or without treatment, were seeded in 6-well plates (1×10^6 cells/well) to reach subconfluence. The cell monolayer was scratched using a pipette tip to create a cell-free gap. The cells were washed with PBS and were cultured in a fresh medium without fetal bovine serum. Wound healing ability was subsequently assessed by measuring the percentage of closure at 0, 24, and 48 h. The experiment was independently repeated thrice.

Trophoblast adhesion assay

The "embryo-endometrial" adhesion model was constructed as previously described with partial improvement.[32] Ishikawa cells, widely used to investigate endometrial receptivity and embryo adhesion, were used in this study. Primary HEECs were isolated from the MS-phase endometrium of the women in the control group, as described above. Primary HEECs were not passaged and used at p0 to reduce the likelihood of stromal cell contamination. Endometrial epithelial cells were then seeded onto the collagen-coated 24-well culture plates in 30 μL droplets of DMEM/F12 medium under paraffin oil and grown to confluence. Next, shRNA knockdown or plasmid overexpression of ACTN1 was performed.

Hatched blastocysts with normal morphology, obtained from C57BL/6 mice, were subsequently added to the treated Ishikawa cells or primary HEEC-confluent monolayers in G-2 plus media (Vitrolife, Gothenburg, Sweden). The co-cultured cells were incubated at 37°C for 6 h, followed by two washes with PBS. Adherent blastocysts were imaged using a fluorescence microscope (Nikon, TS, Japan). The experiment was repeated at least thrice.

In vivo experiment

The 8-week-old C57BL/6 female mice purchased from Vital River Laboratory Animal Technologies Co. Ltd. were divided into two group, namely, the Nc and ACTN1-sh groups. Thereafter, the mice each in group were administered 20 μL of concentrated lentivirus solution (10^8 units/mL) via injection into uterine horns via the vagina using a flat-head microsyringe. The lentivirus, purchased from Hanbio company

(Shanghai, China), was characterized by a green fluorescent protein (GFP) coding sequence. This treatment was performed at twice after every 2 days. Then, 2 days after the last injection, the mice were sacrificed and their uterus were collected for analysis. All animal experiments were carried out in accordance with the guidelines for the Use of Animals in Research issued by the Shanghai Jiao Tong University, School of Medicine.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

Statistical analysis was performed using a two-tailed Student's *t* test to calculate the differences between the two groups. For more than two groups, a one-way analysis of variance with either the Bonferroni or nonparametric test was performed. Statistical analyses were performed using SPSS software (version 22.0; IBM, Armonk, NY, USA). Differences were considered statistically significant at $p < 0.05$.